IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

Marcus B. Gohlke

Serial No.: 10/021,970

Filed: December 13, 2001

For: COMPOSITIONS CONTAINING BETA-GLUCAN AND LACTOFERRIN AND THEIR USE

Group Art Unit: 1651

Examiner: Susan Coe

Atty Docket: 13479.0002.CPUS01

DECLARATION OF MARCUS B. GOHLKE

Commissioner for Patents
Washington, D.C. 20231

I, Marcus B. Gohlke, of Houston, Texas hereby declare as follows:

1. I am the named inventor on the above described patent application.

2. I have read the Office Action issued by Examiner Susan Coe on February 8, 2002. This Office Action rejected pending claims 1-18 as being unpatentable under 35 U.S.C. § 103(a) in light of U.S. Patent Nos. 5,296,464 ("the '464 patent"), 5,783,569 ("the '569 patent"), and 5,670,138 ("the '138 patent"). The Examiner cited the '464 patent as teaching the use of lactoferrin to treat bacterial infections, and cited the '569 patent as teaching the use of beta glucan to treat bacterial infections.

3. The Examiner indicated that it is obvious to combine two or more ingredients each of which is taught by the prior art to be useful for the same purpose in order to form a third composition which is useful for the same [illegible]

4. For the following reasons, I strongly believe that it is not obvious to combine lactoferrin and beta-glucan as described in the pending claims

5. Beta glucan is known to have bactericidal qualities. When administered to humans, beta glucan activates macrophages, but also dramatically increases TNF-α (tumor necrosis factor α) levels. TNF-α is a cytokine that produces inflammation. In a white paper, *Zasshi Yakugakui*, Structure and biological activities of fungal beta-1,3-glucans, 2000 May;120(5):413-31 (copy attached), Dr. Yakugakui teaches that while some of beta glucan's activities are beneficial and pharmacologically useful (such as interferon-gamma and colony stimulating factor syntheses), other properties are strongly related to allergic and inflammatory adverse reactions. One can reasonably conclude that the adverse inflammatory response is due in large part to the increase in TNF-α. This assumption was confirmed by Gyorgy Abel et al., Stimulation of Human Monocyte Beta Glucan Receptors by Glucan Particles Induces Production of TNF-α and IL-1β, *International Journal of Immunopharmacology*, 1992, Vol. 14, No. 4, pp. 1363-1373 (copy attached). Table 1 shows that monocyte monolayers treated with glucan had TNF-α levels of 2.10 ± 0.90 ng/10^6 cells, while monolayers treated with buffer had TNF-α levels of 0.02 ± 0.02 ng/10^6 cells (an increase of 105 fold). Figures 3-6 show the time-dependent and dosage-dependent effects of glucan particles on TNF-α production

6. Lactoferrin is also known for its bactericidal qualities. When lactoferrin is administered at the rate of 10 mg. per day to humans, a 120% increase in fresh neutrophils in circulation (8.6 + 0.53 over 3.9 ± .50) was observed one day after the last dose (Michael Zimecki

Archivum Immunologiae et Therapiae Experimentalis, 1999, 47, 113-118, (copy attached)). However, in this same study, lactoferrin decreased TNF-α levels in the body by 82% (from 26.1 ± 2.03 to 4.6 ± 1.4) and decreased another inflammation producing cytokine, Interleukin 6 (IL-6), production by 90.4% (from 60.5 ± 6.2 to 5.8 ± 3.5) during the same period.

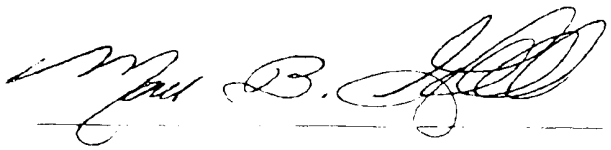
7. The invention described in the pending claims allows the benefit of mitigating the negative side effects of either substance by itself while balancing TNF-α levels resulting in enhanced infection fighting capability with activated macrophages and increased neutrophil levels. Taking only one component (such as beta glucan) results in hyper activated macrophages and substantially increased TNF-α levels resulting in inflammation, without the benefit of increased neutrophils in circulation. Increasing lactoferrin intake results in increased neutrophils in circulation while decreasing TNF-α levels. Lactoferrin taken alone provides a short term benefit for infections and the reduction of inflammation, but with a greater risk of tumor development due to the decrease of TNF-α. This invention allows the continued benefits of activated macrophages, increased neutrophils while mitigating the negative side effects of either substance by itself and balancing TNF-α levels for normal function of sustained tumor eradication.

8. Furthermore, the invention enhances the ability of the body to use neutrophils and macrophages in tandem. Neutrophils seek out metabolic insults, such as bacteria, viruses, and fungi. When these insults are identified, the membrane of the neutrophil ruptures and lactoferrin is expelled. The lactoferrin surrounds the insult and

mentioned, macrophages are activated with beta glucan. A synergistic effect of taking beta glucan with lactoferrin is to allow the body to balance its microbial insult attack of neutrophils and activated macrophages.

9. Contrary to the Examiner's assertion that it would be obvious to combine lactoferrin and beta-glucan since both are known anti-bacterial agents, this invention involves the intellectual step of selecting two components in order to obtain synergistic beneficial effects while balancing TNF-α levels in the body. This combination of components is non-obvious, and leads to a combination of effects and efficacies (reflected in the specification and in originally filed claims 19-24) that are also non-obviousness.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.



Marcus B. Gehlke

August 7, 2002

Date

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Yakugaku Zasshi 2000 May;120(5):413-31

[Structure and biological activities of fungal beta-1,3-glucans].

[Article in Japanese]

Yadomae T

Laboratory for Immunopharmacology of Microbial Products, School of Pharmacy, Tokyo University of Pharmacy and Life Science, Japan.

This paper summarizes the structure, biological activities, signaling, and metabolic degradation of fungal beta-1,3-glucans. Fungal beta-glucans exist both soluble and particulate forms. Conformation of the soluble beta-glucan was classified into three groups: triple helix, single helix and random coil. Fungal beta-1,3-glucans exhibit a variety of biological and immunopharmacological activities, and the significance of these activities is dependent on the structure, such as solubility in water, molecular weight, degree of branching, and conformation. Many of the activities, such as nitrogen oxide synthesis of macrophage and limulus factor G activation, were dependent on the conformation, but some of the others were independent, such as interferon-gamma and colony stimulating factor syntheses. **These activities are beneficial and pharmacologically useful, while some strongly related to allergic and inflammatory adverse reactions**. It should be noted that the beta-glucans, once administered into the body, remain mainly in the liver and spleen for a very long period of time. The activity, at least in part, is maintained during these periods. The biochemical mechanisms of the beta-glucan, especially in soluble form, mediating biological activity was still not clearly demonstrated.

Publication Types:

- Review
- Review, tutorial

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STIMULATION OF HUMAN MONOCYTE β-GLUCAN RECEPTORS BY GLUCAN PARTICLES INDUCES PRODUCTION OF TNF-α AND IL-1β

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(*Received* 20 *April* 1992 *and in final form* 22 *June* 1992)

Abstract — β-glucans are pharmacologic agents that rapidly enhance host resistance to a variety of biologic insults through mechanisms involving macrophage activation. To determine whether stimulation of the β glucan receptors on human monocytes resulted in cytokine production, monolayers of monocytes were incubated with purified yeast glucan particles and measured for tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) mRNA and protein. By Northern blot analysis, TNF-α mRNA was detected within 30 min of incubation with glucan particles, peaked at 2 h, and remained elevated for at least 8 h. Glucan induction of IL-1β mRNA followed a similar time-course of initiation and accumulation. By enzyme-linked immunosorbent assays (ELISAs), significant levels of TNF-α and IL-1β were present in supernatants of glucan-treated cells within 1 h and plateau levels of both cytokines were approached within 4 h. At particle-to-cell ratios of from 0.4 to 18, glucan particles induced dose-dependent increases in TNF-α and IL-1β mRNA and corresponding increases in TNF-α and IL-1β proteins. Exposure of monocytes to glucan particles for 0–30 min and washing before continued incubation for 4 h in particle-free buffer induced production and secretion of TNF-α and IL-1β in a time-dependent fashion compatible with phagocytosis. The pre-treatment of monocyte monolayers with trypsin reduced glucan-induced production of TNF-α and IL-1β in a dose-dependent manner with 5 μg/ml of trypsin effecting reductions of greater than 50%. Thus, glucan particles induce human monocyte production of TNF-α and IL-1β by a mechanism that is dependent on trypsin-sensitive β-glucan receptors.

Glucan particles are carbohydrate polymers derived from the cell walls of *Saccharomyces cerevisiae* and composed solely of β-D-glucose residues with 1,3 and 1,6 linkages (Manners, Masson & Patterson, 1973); yeast glucan is the major constituent of zymosan particles (Di Carlo & Fiore, 1958; Riggi & Di Luzio, 1961). The administration of purified yeast glucan to laboratory animals rapidly gives rise to an augmented state of host defense by mechanisms involving macrophage activation (Di Luzio, Pisano & Saba, 1979). Glucan particles increase host resistance to a diverse range of microbial pathogens (Reynolds *et al.*, 1980), promote the regression of certain tumors (Di Luzio, Williams, McNamee, Edwards & Kitahama, 1979), and improve survival after irradiation by reducing opportunistic infections to endogenous organisms of normal microflora (Patchen, D'Alesandro, Brook, Blakely & MacVittie, 1987) and stimulating hematopoiesis (Patchen & MacVittie, 1985). The protective effects of glucan particles against such biologic insults is likely to involve the rapid generation of cytokines. Production of bioactive tumor necrosis factor (TNF) and interleukin-1 (IL-1) is enhanced in cultures of mouse splenic macrophages by particulate yeast glucan (Sherwood, Williams & Di Luzio, 1986). Intravenous injection of mice or rats with soluble yeast glucan gives rise to substantial levels of circulating granulocyte–macrophage colony stimulating factor (Patchen & MacVittie, 1986), IL-1, and interleukin-2 (IL-2) (Sherwood, Williams, McNamee, Jones, Browder & Di Luzio, 1987) within 1–12 h post-injection.

Human monocytes (Czop & Austen, 1985a), human alveolar macrophages (Czop, McGowan & Center, 1982), human neutrophils (Czop, Puglisi,

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Miorandi & Austen, 1988), and murine macrophages (Kadish, Choi & Czop, 1986; Goldman, 1988) possess trypsin-sensitive β-glucan receptors that initiate rapid phagocytosis of glucan and zymosan particles. The β-glucan receptors on human monocytes have been isolated recently (Czop & Kay, 1991) and have been best characterized in terms of their ligand specificity for β-glucans with 1,3 and/or 1,6 linkages (Czop & Austen, 1985a). In response to glucan or zymosan particles, monocyte β-glucan receptors of comparable trypsin sensitivity and ligand specificity are activated and induce the cells to generate leukotrienes B_4 and C_4 (Czop & Austen, 1985b) and to secrete lysosomal enzymes (Janusz, Austen & Czop, 1987). Little, however, is known about the production of cytokines during the activation of monocyte β-glucan receptors. Heat-killed *S. cerevisiae* opsonized with IgG have been shown to enhance the expression of TNF-α and IL-6 mRNAs in human monocytes (Bazzoni, Cassatella, Laudanna & Rossi, 1991), but the contributions of Fcγ and β-glucan receptors were not examined. Aminated forms of soluble curdlan, a linear β-(1,3)-D-glucan derived from *Alcaligenes faecalis*, have been shown to induce human monocyte production of bioactive TNF-α and IL-1 (Doita, Rasmussen, Seljelid & Lipsky, 1991). In the current paper, we studied the effects of purified yeast glucan particles on human monocyte gene expression of TNF-α and IL-1β and the production of these two cytokines in the same cells. We also sought to determine whether glucan particle induction of monocyte TNF-α and IL-1β exhibited the same time-course of activation and sensitivity to trypsin as β-glucan receptor-mediated phagocytosis.

EXPERIMENTAL PROCEDURES

Materials

General chemicals were obtained from Sigma Chemical Co., St. Louis, MO. Culture reagents and solutions were monitored for endotoxin by the chromogenic *Limulus* amoebocyte lysate (LAL) assay (M. A. Bioproducts, Walkersville, MD); none contained detectable levels of endotoxin (<3 pg/ml).

[illegible] the [illegible] glucan [illegible] particles, [illegible] carbohydrate, [illegible]% protein, and no neutral sugar other than glucose. By the coagulation LAL assay (M. A. Bioproducts), stock suspensions of glucan particles contained no detectable endotoxin (<3 pg/10^8 particles).

Preparation of monocyte monolayers

Human peripheral blood mononuclear cells were isolated (Janusz *et al.*, 1987) from normal citrated and dextran-treated blood, washed free of plasma and platelets in Hank's balanced salt solution (HBSS) lacking calcium, magnesium, and phenol red, and purified by gradient centrifugation on cushions of Ficoll–Paque (Pharmacia Fine Chemicals, Piscataway, NJ). The mononuclear cells were collected into HBSS, washed twice, resuspended in RPMI 1640 Medium (Gibco, Grand Island, NY) containing 1% heat-inactivated autologous serum (56°C for 30 min), and counted on the Coulter counter.

For the preparation of monocyte monolayers, 1 ml of 2.2×10^6 mononuclear cells/ml was plated into wells of 24-well tissue culture plates (Costar, Cambridge, MA), incubated for 1 h at 37°C in a humidified atmosphere of 5% CO_2, and washed three times with 2 ml of RPMI to remove the nonadherent cells. A second 1-ml aliquot of 2.2×10^6 mononuclear cells/ml was layered into each well and incubated for 2 h as described above before removal of the nonadherent cells. By visual enumeration at 40 × with an inverted phase microscope and a calibrated reticle, the number of adherent cells for 30 different donors was $0.77 \pm 0.20 \times 10^6$ per well (mean ± S.D.). By morphology and nonspecific esterase staining, >95% of the adherent cells were monocytes.

Measurement of TNF-α and IL-1β

Monocyte monolayers were incubated at 37°C in the CO_2 chamber for 0–8 h with 0.5 ml of RPMI, 1% heat-activated autologous serum, 10 mM HEPES, and 5 mM $MgCl_2$ in the absence and presence of the number of glucan particles specified in the text. The culture supernatant was removed, clarified by centrifugation at 14,000 *g* for 5 min at 4°C, and stored at −70°C before assay of cytokines.

[illegible] supernatants was determined with the Cistron II [illegible] ELISA [illegible]

Fig. 1. Northern bl[ot] glucan particles (G[lucan] monocytes, the em[ount] before blotting and

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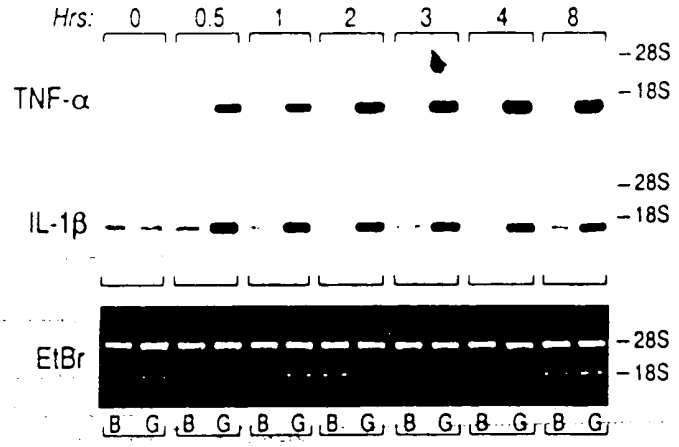

Fig. 1. Northern blot analysis of mRNA for TNF-α and IL-1β in human monocytes incubated with buffer (B) and 2 × 10^7 glucan particles (G) for 0–8 h. Total cellular RNA was isolated from individual monolayers containing 6.9 × 10^5 monocytes, the entire RNA sample was size-separated by gel electrophoresis and stained with ethidium bromide (EtBr) before blotting and probing. The data are representative of four studies with different monocyte donors. Mobility of 28S and 18S ribosomal RNA are indicated.

which had a lower limit of detectability of 25 pg/ml. The data are expressed as nanograms per 10^6 monocytes, which was calculated by dividing the quantity of cytokine in 0.5 ml of supernatant by the number of monocytes per well.

Cell-associated levels of TNF-α and IL-1β were determined for newly prepared monocyte monolayers. For these determinations, the cells were lyzed in 0.25 ml PBS by three rounds of freezing and thawing, the lyzates were cleared of debris by centrifugation at 14,000 *g* for 5 min at 4°C, and the resulting supernatants were stored at −70°C. Newly prepared monocyte monolayers contained no detectable levels of intracellular TNF-α and no significant levels of intracellular IL-1β (0 – 10 pg/10^6 cells).

RNA isolation and Northern blot analysis

Total cellular RNA was isolated (Chomczynski & Sacchi, 1987) from individual wells of buffer- and glucan-treated adherent monocytes, resolved by electrophoresis in 1.2% agarose gels containing 6% formaldehyde, and visualized by staining with ethidium bromide. The RNA was transferred to nylon membranes (Micron Separations, Inc., Westboro, MA) in 20 × SSC (3 M sodium chloride and 0.3 M sodium citrate, pH 7) by capillary action and crosslinked to the membrane by shortwave ultraviolet radiation. The blot was pre-hybridized at 42°C for 18 h in 0.1% sodium dodecyl sulfate (SDS), 50% formamide, 2.5 × Denhardt's solution (0.05% Ficoll, 0.05% bovine serum albumin (BSA), 0.05% polyvinyl pyrrolidine), 5 × SSC, 10 mM sodium phosphate (pH 7.0), 2 mM EDTA, and 0.1 mg/ml boiled herring sperm DNA.

The blot was probed in pre-hybridizing solution containing 10% dextran sulfate and cDNA probes that had been radiolabeled with (α-^{32}P)dCTP (3000 Ci/mmol) (Amersham Corp., Arlington Heights, IL) by random priming (Amersham). The specific activity of the radiolabeled cDNA probes was 3 – 8 × 10^8 counts/min/μg. The TNF-α probe (Oliff *et al.*, 1987) was a 600-bp, Hind III – EcoR I fragment of the full-length cDNA subcloned in pGEM3 (Merck, Sharp, and Dohme Research Laboratories, Rahway, NJ). The IL-1β probe was a 1.3-kb Pst I fragment of the IL-1β precursor subcloned in pSP64 (Genetics Institute, Cambridge, MA).

The hybridized blots were washed twice at 25°C in 0.1% SDS 2 × SSC and twice at 65°C in 0.1% SDS 0.2 × SSC, air-dried, and exposed to X-ray film (XAR-5, Eastman Kodak, Co., Rochester, NY).

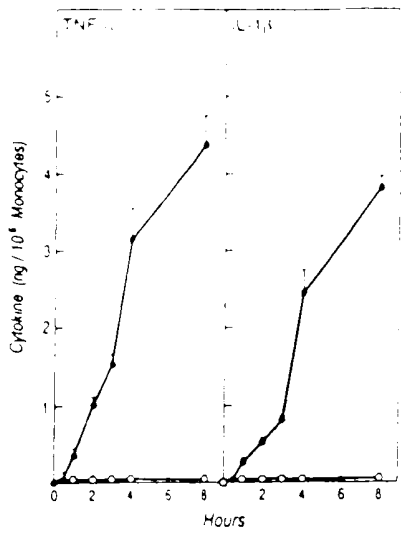

Fig. 2. Time-dependent effects of glucan particles (●) and buffer (○) on monocyte production of TNF-α and IL-1β. The quantities of immunoreactive TNF-α (left panel) and IL-1β (right panel) were measured in culture supernatants of the monocyte monolayers depicted in Fig. 1. The data are expressed as nanograms per 10^6 monocytes, are shown as the mean with range for duplicate determinations of duplicate monolayers, and are representative of four studies with different donors.

radioautographs were scanned by densitometry to quantitate the relative levels of cytokine mRNA.

Trypsin treatment

Monolayers of monocytes were treated with 1 – 50 μg/ml of affinity-purified trypsin (Czop, Fearon & Austen, 1978) in RPMI for 20 min at 37°C, washed, incubated with 1 μg/ml of trasylol in RPMI for 5 min at 37°C, and washed. The pre-treated monolayers were incubated, as described above, with 0.5 ml of buffer or glucan particles, and subsequently assessed for cytokine production. Trypsin pre-treatment had no effect on the number of monocytes adhering to the wells.

RESULTS

Kinetics of cytokine transcription and translation by glucan-stimulated monocytes

Monolayers of human monocytes were incubated at 37°C for 0 – 8 h with buffer alone and containing 2 × 10^7 glucan particles. The culture supernatant

Table 1. Range of monocyte levels of TNF-α and IL-1β in response to buffer and glucan particles*

Donor	TNF-α ng/10^6 cells		IL-1β ng 10^6 cells	
	Buffer	Glucan	Buffer	Glucan
1	0.03	4.14	0.01	0.72
2	0.01	3.02	0.08	0.95
3	0.01	2.79	0.05	0.35
4	0.01	2.47	0.04	1.95
5	0.03	2.14	0.04	3.34
6	0	2.00	0	0.30
7	0.01	1.98	0.02	0.54
8	0.01	1.82	0.08	1.21
9	0.06	1.40	0	0.09
10	0	1.24	0.04	1.25
11	0.03	1.23	0.03	0.41
12	0.02	0.98	0	1.80

*Monocyte monolayers from 12 different donors were incubated for 4 h in the absence and presence of 2×10^7 glucan particles. Cell-free culture supernatants from duplicate monolayers were assayed in duplicate by cytokine-specific ELISAs.

RNA was analyzed in Northern blots for TNF-α and IL-1β mRNA. Monocytes exhibited rapid kinetics in TNF-α and IL-1β gene expression in response to glucan particles (Fig. 1). The numbers of adherent monocytes, as assessed by direct counting and by the levels of cellular RNA stained with ethidium bromide, were unchanged by prolonged incubation or glucan particles. Monocyte synthesis of TNF-α mRNA was induced within 30 min of incubation with glucan particles and this was followed by an increase of 2-fold at 2 h, which remained unchanged for at least 8 h. Monocytes cultured in buffer without glucan particles synthesized no detectable TNF-α mRNA regardless of the time of incubation. Synthesis of IL-1β mRNA by the same donor monocytes reached maximum steady-state levels within 30 min of stimulation with glucan particles and these levels continued to be expressed for at least 1 h. Unlike TNF-α mRNA, IL-1β mRNA declined 2-fold by 4 h and remained at this level for at least 8 h. In two of four experiments, monocytes cultured in buffer alone contained an initial content of IL-1β mRNA which progressively decreased over the 8-h time interval. In two of the experiments, buffer-cultured monocytes contained no detectable levels of TNF-α or IL-1β mRNA [illegible]

monocyte secretion of TNF-α and IL-1β proteins, whereas buffer alone did not (Fig. 2). Significant quantities of monocyte TNF-α and IL-1β were both detected after 1 h of incubation with glucan particles. At this time point, monocytes produced 0.39 ng of TNF-α and 0.27 ng of IL-1β per 10^6 monocytes, respectively. These quantities were increased after 4 h to values of 3.16 ng of TNF-α and 2.44 ng of IL-1β per 10^6 cells.

To determine the ranges in concentrations of secreted TNF-α and IL-1β, duplicate monolayers from 12 separate donors were incubated for 4 h at 37°C with buffer or 2×10^7 glucan particles. The quantities of TNF-α secreted by glucan-stimulated monocytes ranged from 0.98 to 4.14 ng per 10^6 monocytes with a mean ± S.D. of 2.10 ± 0.90 ng/10^6 cells; the quantities of secreted IL-1β ranged from 0.09 to 3.34 ng per 10^6 monocytes with a mean ± S.D. of 1.08 ± 0.93 ng/10^6 cells (Table 1).

Dose effects of glucan particles on monocyte production of TNF-α and IL-1β

Incubation times of 1, 2, and 4 h were selected to follow [illegible] glucan particles, which corresponded to particle-to-cell ratios of 0, 0.4, 0.7, 1.8, and 18, respectively. Monocyte [illegible] stimulated time-dependent increases in [illegible]

Fig. 3. Dose – resp
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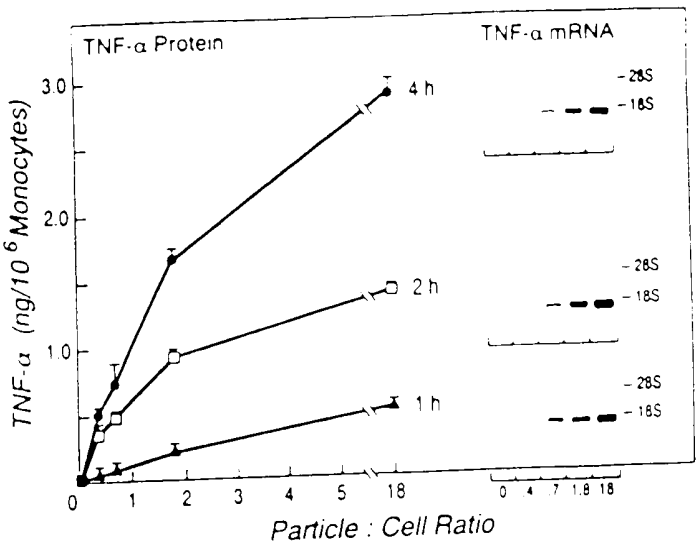

Fig. 3. Dose – response effects of glucan particles on mRNA induction and translation of TNF-α. Monolayers with 1.12×10^6 monocytes were incubated with increasing numbers of glucan particles for 1 (▲), 2 (□), and 4 (●) h. The levels of immunoreactive TNF-α (left panel) and TNF-α mRNA (right panel) were determined in culture supernatants by ELISA and in preparations of isolated cellular RNA by Northern blot analysis, respectively. The data for TNF-α protein are expressed as nanograms per 10^6 monocytes and are presented as the mean with range of duplicate determinations of duplicate monocyte monolayers. The data are representative of three studies with different donors. Mobility of 28S and 18S ribosomal RNA are indicated.

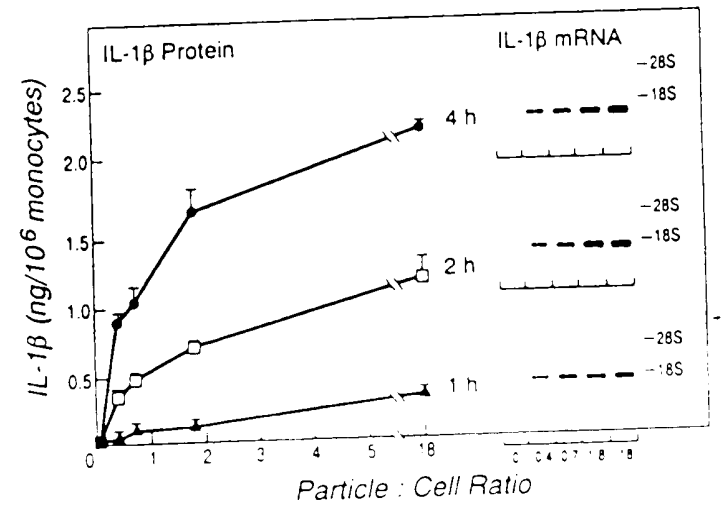

Fig. 4. Dose – response effects of glucan particles on mRNA induction and translation of IL-1β after 1 (▲), 2 (□), and 4 (●) h. Immunoreactive IL-1β (left panel) and mRNA for IL-1β (right panel) were measured in culture supernatants and Northern blots of isolated monocyte RNA, respectively. The data for IL-1β protein are expressed as nanograms per 10^6 monocytes and are plotted as the mean with range for duplicate determinations of duplicate monocytes. The data are representative of three studies with different donors and the samples are from the same experiment depicted in Fig. 3. Mobility of 28S and 18S ribosomal RNA are indicated.

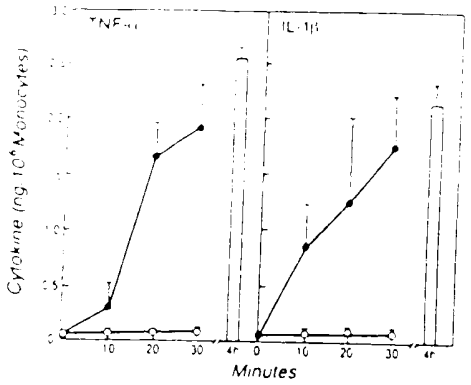

Fig. 5. Time-dependent effects of glucan particles on subsequent production of TNF-α and IL-1β by cultured monocytes. Monolayers of monocytes were incubated for various times with buffer (○) and 2×10^7 glucan particles (●), washed, and cultured in particle-free buffer for 4 h. The levels of immunoreactive TNF-α (left panel) and IL-1β (right panel) in culture supernatants were measured by ELISA; similar measurements were made for monolayers incubated for 4 h with the same number of glucan particles. The data are expressed as nanograms per 10^4 monocytes, are shown as the mean with range for duplicate determinations of duplicate monolayers from two different donors, and are representative of three separate studies.

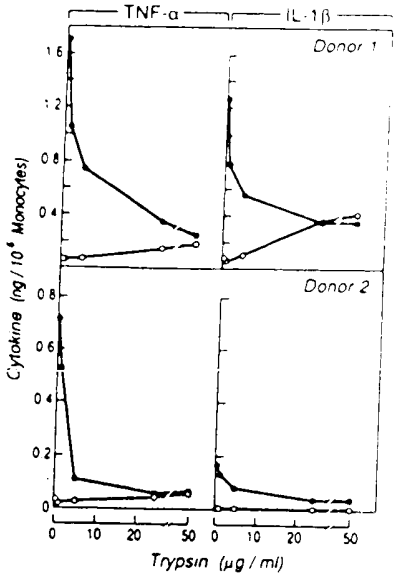

Fig. 6. Dose-dependent effects of trypsin pre-treatment of monocytes on glucan-induced production of TNF-α and IL-1β. Trypsin-treated monocyte monolayers from donors with high (Donor 1) and low (Donor 2) capacities to produce TNF-α and IL-1β were incubated with buffer (○) and 2×10^7 glucan particles (●) for 40 min, washed, and cultured in particle-free buffer for 4 h. The levels of TNF-α (left) and IL-1β (right) in culture supernatants were measured by ELISA. The data are expressed as nanograms per 10^4 monocytes, are plotted as averages of duplicate monolayers for two donors, and are representative of four studies with different donors.

transcription and translation of TNF-α were induced in a dose-dependent fashion by glucan particles but not by buffer alone (Fig. 3). Within 1 h of incubation, the levels of monocyte TNF-α mRNA induced by glucan particles at particle-to-cell ratios of 0.7, 1.8 and 18 were 2-, 4-, and 12-fold greater than that induced by 0.4 particles per cell. Monocyte TNF-α mRNA was further increased by the two higher doses of glucan particles by approximately 2-fold within 2 h. At each time point, the quantities of secreted TNF-α protein were related to the numbers of glucan particles and the levels of TNF-α mRNA. After 4 h of incubation, glucan particles at particle-to-cell ratios of 0, 0.4, 0.7, 1.8, and 18 stimulated monocytes to produce and secrete 0, 0.50, 0.75, 1.67, and 2.88 ng of TNF-α per 10^6 cells, respectively.

The dose effects of glucan particles on inducing monocyte IL-1β were similar to the induction of TNF-α except for a more gradual increase in IL-1β [illegible] higher doses of glucan particles, and was unchanged after incubation for 2 and 4 h with the two lower doses. At each incubation time, monocyte secretion of IL-1β protein was related to the input of glucan particles and to the level of IL-1β mRNA. After 4 h, monocytes that had been incubated with 0, 0.4, 0.7, 1.8, and 18 glucan particles per cell secreted 0, 0.93, 1.05, 1.72, and 2.18 ng of IL-1β per 10^6 cells, respectively.

Monocyte secretion of TNF-α and IL-1β after brief exposure to glucan particles

Monocyte phagocytosis of glucan particles occurs [illegible] as observed with TNF-α mRNA, IL-1β mRNA was increased at 2 h by about 2-fold by the two [illegible] glucan particles, rinsed free of noningested particles, and assessed for cytokine secretion after incubation for 4 h in buffer [illegible]

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activated within 10 – 20 min by glucan particles in their capacities to generate TNF-α and IL-1β (Fig. 5). After incubation for 30 min with glucan particles and culture for 4 h in particle-free buffer, monocytes secreted TNF-α and IL-1β in quantities that were 75 and 82% of those produced by monocytes incubated for 4 h with particles. In the absence of culture, monocytes treated for ≤30 min with glucan particles secreted TNF-α and IL-1β at levels which were near or below the lower limits of sensitivity of the ELISA assays (data not shown).

Monocyte phagocytosis of glucan particles was assessed for stained preparations of fixed monolayers (Czop *et al.*, 1978) in the tissue culture plates with an inverted light microscope at 400 ×. For two donors, the average percentages of monocytes ingesting ≥1 and ≥3 particles, after 30 min, were 82 and 55%, respectively. After 4 h of culture in particle-free buffer, the average percentages of monocytes ingesting ≥1 and ≥3 particles were 82 and 50%, respectively, and these values corresponded to 83 and 54% for cells incubated for 4 h with glucan particles.

Effect of trypsin-pre-treatment of monocytes on glucan-induced secretion of TNF-α and IL-1β

Monolayers of monocytes from donors who exhibited high and low capacities to produce TNF-α and IL-1β were treated for 20 min with 1 – 50 μg/ml of affinity-purified trypsin, washed, incubated with 1 μg/ml of trasylol, and washed again. The cells were stimulated for 40 min with 2 × 10^7 glucan particles, washed free of noningested particles, and assessed after a 4 h incubation in buffer for TNF-α and IL-1β. Glucan-induced cytokine production and secretion was markedly reduced by trypsin-pre-treatment of monocytes from donors with high and low capacities to generate TNF-α and IL-1β (Fig. 6). For the two types of donor monocytes, pre-treatment with 5 μg/ml of trypsin decreased glucan-induced secretion of TNF-α by 61 and 88%; the corresponding decreases in IL-1β were 56 and 57%. Trypsin concentrations of ≥25 μg/ml further decreased the glucan-induced responses to levels of the buffer controls.

DISCUSSION

Human monocytes rapidly phagocytose glucan particles through β-glucan receptors that are exquisitely sensitive to inactivation by trypsin (Czop *et al.*, 1978; Czop & Austen, 1985a). The present study demonstrates that the stimulation of monocyte β-glucan receptors by glucan particles results in the transcription and translation of TNF-α and IL-1β. By Northern blot analysis, transcription of TNF-α and IL-1β genes was rapidly induced by glucan particles as indicated by the detection of substantial amounts of cellular cytokine-specific mRNAs within 30 min of activation (Fig. 1). TNF-α and IL-1β mRNAs were each presented as a single distinct band that exhibited little or no degradation by monocytes cultured for as long as 8 h with glucan particles. The sizes of glucan-induced TNF-α and IL-1β mRNAs correspond to typical full-length mRNAs of approximately 1.8 kb for TNF-α (Goeddel *et al.*, 1986) and 1.6 kb for IL-1β (Webb, Auron, Rich, Rosenwasser, Wolff & Dinarello, 1985).

In our studies, adherence-mediated stimulation of monocyte TNF-α (Eierman, Johnson & Haskill, 1989) and IL-1β (Schindler, Clark & Dinarello, 1990) mRNAs were largely diminished by the methods used to prepare and culture monocyte monolayers. No detectable levels of TNF-α mRNA were present in monocytes cultured in media devoid of particles (Figs 1 and 3); however, low levels of IL-1β mRNA were detected in monolayers from some (Fig. 1) but not all (Fig. 4) monocyte donors. Induction of TNF-α mRNA by glucan particles began within 30 min of incubation, peaked by 2 h, and remained elevated for at least 8 h (Fig. 1). Glucan induction of IL-1β mRNA followed a similar time-course of initiation, accumulation, and apparent stabilization. The absence of a differential effect on these two cytokines was further observed in dose – response experiments in which mRNAs for TNF-α (Fig. 3) and IL-1β (Fig. 4) were both induced in monocyte monolayers by fewer than 1 to as many as 18 glucan particles per cell.

The induction of mRNA transcripts by glucan particles was followed by time- (Fig. 2) and dose-dependent (Figs 3 and 4) increases in the production and secretion of both TNF-α and IL-1β protein. As with TNF-α and IL-1β mRNAs, glucan particles exhibited no differential time- or dose-related effects on the quantities of TNF-α or IL-1β that were produced and secreted. Although TNF-α accumulated in the culture medium at higher concentrations than IL-1β, monocyte production and secretion of both TNF-α and IL-1β approached plateau levels at the same time of incubation and at the same input of glucan particles. The primary stimulatory events, namely those involving contact and occupation of monocyte β-glucan receptors with glucan particles, occurred within 10 – 20 min of incubation and were of the same short duration as a phagocytic response

(Fig. 5). Monocytes containing ingested glucan particles, however, produced no TNF-α or IL-1β unless they were subsequently cultured for a time interval compatible with protein synthesis. Under nearly identical conditions of assay, the action of glucan particles on inducing monocyte release of preformed metabolites is considerably more rapid. Significant levels of lysosomal enzymes are released by monocytes within 30 min of incubation with glucan particles and these values reach maximal levels within 1 h (Janusz *et al.*, 1987).

The use of monocyte monolayers that were essentially free of other cell types and their stimulation with preparations of pure glucan particles limited the ligand – receptor interactions involved in cytokine production to those mediated by monocyte β-glucan receptors. That activation of β-glucan receptors was sufficient to transmit the signals necessary for cytokine production was further indicated in studies with trypsin-treated monocytes. The pre-treatment of monocytes with 1 – 50 μg/ml of trypsin reduced glucan particle induction of TNF-α and IL-1β in a dose-dependent fashion with 5 μg/ml of trypsin effecting reductions of over 50% (Fig. 6). These reductions in cytokine production were virtually identical to the trypsin liability of other cellular functions known to be stimulated by monocyte β-glucan receptors, namely phagocytosis (Czop *et al.*, 1978), leukotriene generation (Czop & Austen, 1985b), and lysosomal enzyme release (Janusz *et al.*, 1987), thereby linking each of these functions to a common cell surface protein. Thus, monocyte receptors for yeast β-glucans provide an important physiologic mechanism for initiating particle clearance and inducing post-receptor signals that give rise to a broad spectrum of inflammatory metabolites, which includes TNF-α and IL-1β.

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Lactoferrin Increases the Output of Neutrophil Precursors and Attenuates the Spontaneous Production of TNF-α and IL-6 by Peripheral Blood Cells

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Abstract. The aim of this report was to investigate the effects of bovine lactoferrin (BLF) taken orally (*per os*) by healthy individuals, on selected immune parameters. Three groups of volunteers (7 persons per group) were taken daily for 7 days, one capsule containing 2, 10 or 50 mg of BLF. A control group has taken placebo only. Venous blood was taken for tests a few hours before the first dose of BLF, one day and 14 days after the last dose of the preparation. For the evaluation of BLF action on the immune response system we have chosen 3 parameters: content of neutrophil precursors in the peripheral blood (in percentage), spontaneous production of interleukin 6 (IL-6) and tumor necrosis factor α (TNF-α) by unstimulated blood cell cultures. We found that oral treatment of volunteers with BLF caused a transient (one day after last dose) increase of immature forms of neutrophils in the circulating blood. That increase was more than 2-fold in the case of 10 mg dose. However, statistically significant increases in the percentage of neutrophil precursors were also registered at doses of 2 and 50 mg of BLF. No change in the immature cell content was observed in the placebo group. The treatment with BLF also resulted in a profound decrease of the spontaneous production of IL-6 and TNF-α by cultures of peripheral blood cells. This decrease was significant (10 mg/dose) one day following the last dose of BLF and persisted for additional 14 days. These results confirmed our earlier data on the effects of *per os* treatment with a nutritional preparation containing BLF. Furthermore, we were able to closer establish the optimal dose of BLF affecting selected immune indices.

Key words: bovine lactoferrin; blood cells; neutrophil precursors; interleukin 6; tumor necrosis factor α.

Introduction

Despite enormous progress in modern medicine, immune response to surgery, or its excessive values are usually undesirable[1,2]. Attempts to alleviate uncontrolled immune response in patients by

antiinflammatory compounds can diminish clinical manifestation of the disease, however often significant toxicity associated with such a therapy, prevents from common use of those compounds.

It is, therefore, a great interest to develop a new modality that would apply natural immunomodulators which are constitutive parts of our physiological system. In our laboratory we have studied potential clinical application of an extract from calf thymus[1] and a proline-rich polypeptide from ovine colostrum[2, 23]. Recently, we also turned our attention to lactoferrin (LF), an iron-binding protein contained in milk and secretory fluids of mammals (for review see[4]). Specific receptors for LF have been described on many cell types including brush border cells[15] and monocytes[17]. LF was shown to exhibit antibacterial, antiviral, antifungal, antiparasite and antitumor properties[3, 22, 24, 25, 29]. We and others have demonstrated its effects on maturation of lymphocytes[32, 35] and cytokine production[5, 7, 36]. More recently we concentrated our research on potential application of LF in prevention and therapy. BLF was found to attenuate surgery-elicited cytokine production in mice[30], and modified selected immune parameters in surgery, trauma and septic patients *in vitro*[7, 30]. BLF is frequently found as an ingredient of commercially available nutritional products. These products are recommended in cases of malnutrition, impaired iron metabolism or improper colonization of the intestinal flora[7, 20]. Using one of those nutritional products, Nutrifemme, containing in addition to BLF a number of antioxidants, we found that the preparation, given orally (*per os*), affected several immune parameters in healthy volunteers[38]. Two parameters were preferentially altered – the output of neutrophil precursors into circulation and the ability of blood cell cultures to spontaneously produce IL-6 and TNF-α. Although we regarded BLF to be solely responsible for the observed immunoregulatory effects, a possible influence of other antioxidants such as selenium, dismutase, vitamins E and C could not be excluded.

Therefore, the aim of this study was to confirm the role of BLF, taken orally, in modifying selected immunological parameters. In addition, by using 3 different doses of BLF, we attempted to establish a dose of BLF changing most profoundly the level of neutrophil precursors and spontaneous cytokine production.

The BLF was admixed in capsule with lactose. Placebo capsules contained lactose only.

Treatment of volunteers with BLF. Twenty seven healthy volunteers (11 men and 17 women, age 25–55 years) were divided into 4 groups consisting of 7 individuals each. They were taken *per os* 1 capsule daily, for 7 days, containing 1) placebo (lactose), 2) 2 mg BLF, 3) 10 mg BLF, and 4) 50 mg of BLF. Venous blood was withdrawn into heparinized tubes (5 ml) from each patient at: 1) time 0 (a few hours before the first dose), 2) 1 day after taking the last dose and 3) 14 days following the last dose of BLF.

Preparation of blood cell cultures. Heparinized blood was diluted with RPMI-1640 culture medium to achieve a concentration of 10^6 cells/ml. The cells were distributed in 2 ml aliquots to 24-well culture plates and cultured overnight in a cell culture incubator. The supernatants were harvested and used for cytokine determination.

Determination of IL-6 activity. The assay was performed according to VAN SNICK et al.[28]. Briefly, 7TD1 indicator cells were washed 3 times with Hanks' medium and resuspended in Iscove's medium supplemented with 10% FCS, HEPES buffer, glutamine and antibiotics to a density of 2×10^4 cells/ml. Then, the cells were distributed in 100 µl aliquots into 96-well flat-bottom plates containing 100 µl serially diluted plasma or supernatant in triplicate. After 72 h of culture the proliferation of 7TD1 cells was determined using the MTT colorimetric method[16]. The results of IL-6 activity are presented in pg per ml – such concentration of IL-6 corresponds to the activity of IL-6 expressed in U/ml[28]. One unit of IL-6 activity was calculated as the inverse dilution of a plasma sample where a half-maximal proliferation of 7TD1 cells was registered.

Determination of TNF-α activity. For determination of TNF-α activity the indicator clone WEHI 164.13 was used. The cells were washed 3 times with Hanks' solution and resuspended in RPMI 1640, supplemented with 10% FCS, glutamine and antibiotics at a concentration of 2×10^5/ml. The cells were then distributed into 96-well, flat-bottom plates (2×10^4/well). Serially diluted plasma or supernatant samples were prepared on separate plates and transferred to microtiter plates containing WEHI 164.13 indicator cells. The medium contained in addition 1 µg/ml Actinomycin D to increase sensitivity of the assay. After an over-

Department of Bioorganic Chemistry, Centre of Molecular and Macromolecular Studies, Polish Academy of Sciences, Łódź, Poland). One unit of TNF-α was calculated as an inverse dilution of a given plasma sample where 50% survival of WEHI 164.13 cells took place.

Colorimetric determination of cells proliferation/death. The assay was performed according to Hansen et al.[13]. Briefly, MTT solution, 5 mg/ml in 0.9% NaCl, was added in a volume of 25 µl/well and incubated for 2–4 h. Then, 100 µl of a lysing buffer was added (20% SDS, 50% DMF, pH 4.7). After on overnight incubation at 37°C the optical density (OD) was measured using ELISA reader Dynatech 5000, at the wavelength of 550 nm and reference wavelength of 630 nm.

Morphology of blood cells. A drop of heparinized blood was applied onto a microscopic slide and a smear was made. After drying, the preparation was treated with May-Grünwald reagent and then with Giemsa reagent. The cells were counted (differentiated) at a magnification of 800 × in an immersion oil. Up to 200 cells were counted. The results are presented as a percentage of immature forms of neutrophils.

Statistics. The results were evaluated using Student's *t*-test. The results are presented as a mean values from 7 determinations (individuals) ±SE. The differences were regarded significant when $p>0.05$.

Results

Elevation of immature neutrophil number in the peripheral blood of individuals treated with BLF

Previous study on volunteers, taking a nutritional preparation containing BLF orally, showed that such a treatment resulted in a 2-fold increase in the level of neutrophil precursors in circulation[38]. In this study we presented (Table 1) that treatment of volunteers with various concentration of BLF caused a transient, statistically significant increase in immature neutrophil levels. The highest output of neutrophil into circulation was achieved for patients taking 10 mg BLF/dose. No significant changes in the levels of this cell type were registered in persons receiving placebo only (lactose-containing capsules).

Effect of BLF treatment on the spontaneously produced IL-6 and TNF-α by blood cell cultures

Table 1. Percentage of immature neutrophil forms in the peripheral blood of healthy volunteers taking orally bovine lactoferrin (BLF)

Group	Before treatment	1 day after last dose	14 days after last dose
Placebo	4.0 ± 0.0	4.7 ± 0.55	4.1 ± 0.2
		NS	NS
BLF 2 mg	3.7 ± 0.5	6.4 ± 0.6	4.1 ± 0.05
		p<0.02	NS
BLF 10 mg	3.9 ± 0.5	8.6 ± 0.53	5.6 ± 0.4
		p<0.01	NS
BLF 50 mg	3.4 ± 0.3	5.7 ± 0.4	4.7 ± 0.7
		p<0.05	NS

The volunteers were taking BLF capsules daily for 10 days. The percentages of immature neutrophil forms were determined in blood smears before, 1 day, and 14 days after last dose of BLF. The number of individuals per group = 7

NS – not significant.

Table 2. Spontaneous production of TNF-α by peripheral blood cultures of healthy volunteers taking orally bovine lactoferrin (pg/ml)

Group	Before treatment (1)	1 day after last dose (2)	14 days after last dose (3)	p
Placebo	16.8 ± 2.8	14.4 ± 2.27	19.3 ± 2.9	NS
BLF 2 mg	20.5 ± 1.2	15.7 ± 0.91	14.7 ± 3.5	NS
BLF 10 mg	26.1 ± 2.03	4.6 ± 1.4	3.0 ± 0.9	1:2 <0.001
				1:3 <0.001
BLF 50 mg	14.5 ± 3.7	9.0 ± 3.0	5.4 ± 1.5	1:2 NS
				1:3 <0.001

Spontaneous TNF-α production was determined in 24 h whole blood cultures using the indicator cell line WEHI 164.13.

The significance of the BLF effects after 1 day and 14 days following the last dose was calculated as compared to the initial TNF-α values (1:2 or 1:3)

Table 3. Spontaneous production of IL-6 by peripheral blood cultures of healthy volunteers taking orally bovine lactoferrin (pg/ml)

Group	Before treatment (1)	1 day after last dose (2)	14 days after last dose (3)	p
Placebo	24.6 ± 6.9	29.3 ± 1.6	34.1 ± 3.7	1:2:3 NS
BLF 2 mg	32.5 ± 3.5	26.3 ± 5.2	19.5 ± 7.6	1:2 NS
				1:3 NS
BLF 10 mg	60.5 ± 6.2	7.8 ± 3.5	2.1 ± 0.2	1:2:3 <0.001
BLF 50 mg	25.5 ± 5.2	16.9 ± 6.8	1.6 ± 0.3	1:2 NS
				1:3 <0.001

Spontaneous IL-6 production was determined in 24 h whole blood cultures using the indicator cell line 7TD1.

The significance of the BLF effects after 1 day and 14 days following the last dose was calculated as compared to the initial IL-6 values (1:2 or 1:3)

inhibitory effect correlates with the highest output of neutrophil precursors in the group taking 10 mg BLF/day. The dose of 2 mg caused some, not statistically significant, decreases in the spontaneous production of cytokines. The response to BLF in persons taking 50 mg/day seems to be similar to 10 mg/dose.

Discussion

One of the great advances of modern immunology is the recognition that normal immune homeostasis depends on co-ordinated interactions among the various immune cells. The balance is achieved largely through intracellular communication mediated by a network of cytokines. The production of this highly diverse group of small molecular weight proteins is further controlled by many constituents of the phagocytic cells. Lactoferrin is one of those constituents that is released from the activated neutrophils and plays and important role in a feedback mechanism of inflammatory responses[29]. Although, lactoferrin is often discussed as a mediator of various insult-induced metabolic imbalances, its potential immunoregulatory function has been severely underestimated. In general, lactoferrin is considered as an antimicrobial factor rather than a systemically acting immunomodulator.

In this communication we demonstrated that BLF, taken orally, can significantly alter the immune responses of persons by elevating the percentage of immature neutrophil forms and decreasing the ability of blood cells to spontaneously produce IL-6 and TNF-α. The ability of BLF to increase the turnover of neutrophils shown, in this study, was comparable to that described previously for Nutrifemme, a nutritional supplement containing BLF[38].

It has been suggested that a more rapid turnover of neutrophils is triggered by LF released from degranulating neutrophils following infection or after treatment with LF which simulates infection[1, 4]. This is associated with a transient decrease in neutrophil number[1, 3]. Our unpublished observations showed that in the circulation of septic, non-surviving patients, the level of neutrophil precursors may attain as much as 30%. At the same time the level of released lactoferrin was up to 10 times higher as compared with the physiological concentration. Therefore, it seems logical that ingestion of [illegible] by [illegible] as well by [illegible] the increased turnover production by cells isolated from BLF-treated individuals. The ability of BLF to suppress spontaneous production of cytokines was much higher when compared with the Nutrifemme study[38]. This could be attributed to the different regiment treatment, type of BLF (different source) or final composition of the medication.

The spontaneous production of cytokines may be elicited by minor non-specific[11] and specific stimuli[37]. It is usually 10–20 × lower as compared with LPS-induced cytokine production in PBMC cultures[1, 30]. Explanation of the mechanism, by which orally taken BLF causes suppression of the spontaneously released cytokines, may be at present only speculative. Most likely, BLF by induction of TNF-α[1, 30] and other cytokines[5] may modulate expression of cell receptors responsible for recognition[17], adhesion[33] and activation[34] of cells. In addition, LF is able to activate cells in a manner similar to that of mitogens[8]. Thus, repeated treatment with BLF may induce a state of hyporeactivity. However, when we studied the LPS-induced cytokine production in BLF-treated individuals we only observed a regulatory effect of BLF[38]. These differences may result from the nature of eliciting signals (cell-to-cell, MHC-restricted interactions versus mitogen action).

Nevertheless, the resultant effect of BLF treatment seems to be beneficial for the function of the immune system since it increases by several fold the ratio between LPS-induced versus spontaneous cytokine production[38]. In other words, the relative cell response to exogenous stimuli is stronger. Such a property of the immune system cells would be particularly relevant in an adequate immune response to surgery which is essential to combat potential infections and to accelerate the wound healing process.

In summary, the data presented herein provide essential information with regard to optimal treatment for clinical patients who would benefit from the immunoregulatory properties of lactoferrin. Since only isolated studies described lactoferrin oral effects on phenotype and activity of blood cells[7] or preventive effects in neutropenic patients[27], further studies are clearly needed to elucidate the mechanism by which lactoferrin modulates the immune system.

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